Universidade de Aveiro Departamento de Biologia
2012

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PESQUISA DE MUTAÇÕES NO GENE *inlA* DE *Listeria monocytogenes*

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SEARCH FOR MUTATIONS ON GENE *inlA* OF *Listeria monocytogenes*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo de Microbiologia Clínica e Ambiental, realizada sob a orientação científica do Doutor António Correia, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro, Doutora Paula Teixeira, Professora Auxiliar da Escola Superior de Biotecnologia da Universidade Católica Portuguesa e Doutora Vânia Ferreira, aluna de Pós-doutoramento da Escola Superior de Biotecnologia da Universidade Católica Portuguesa.

Apoio financeiro da FCT no âmbito do projecto PEst-OE/EQB/LA0016/2011

Dedico este trabalho à minha família e amigos.

o júri

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agradecimentos

Este trabalho não teria sido possível sem a cooperação da Escola Superior de Biotecnologia, Centro Regional do Porto da Universidade Católica Portuguesa. Agradeço em especial ao Centro de Biotecnologia e Química Fina (CBQF) e ao Centro de Investigação e Apoio Tecnológico (Cinate) de Microbiologia. Agradeço também ao grupo de Microbiologia Molecular do Instituto de Biologia Molecular e Celular (IBMC), liderado pelo Doutor Didier Cabanes, por todo o apoio prestado na cultura celular, em especial à Teresa Malheiro.
Não posso deixar de agradecer à Universidade de Aveiro pelos 6 anos de formação superior que me proporcionou.

Agradeço à Professora Paula Teixeira por ter aceite ser minha orientadora neste trabalho e agradeço em especial à Vânia Ferreira por tudo o que me ensinou ao longo deste ano e pela enorme ajuda que me prestou.

Ao Rui Magalhães, Luísa, Isabel, Patrícia e Patrícia Guerreiro um enorme obrigado por toda a paciência e disponibilidade que tiveram em me ajudar.

Aos meus colegas de laboratório Lúcia, Ângela, Ricardo, Ana Carvalheira, Ana Castro, Alexandra e Alexandra Pais agradeço o bom ambiente de trabalho, todos os momentos divertidos e agradeço em especial a forma como me receberam no laboratório.

Reconheço a paciência e apoio indispensáveis da Rosa, Catarina, Beta, Mauro, Rafael e Igor durante este último ano.

Agradeço em especial o apoio incondicional dos meus pais António Jorge e Isabel, do meu irmão Rui Jorge e do meu tio Zé durante toda a minha formação.

palavras-chave

Gene internalina A, *Listeria monocytogenes*, ensaio de invasão em células Caco-2, codões stop prematuros.

resumo

Listeria monocytogenes é uma bactéria patogénica de origem alimentar capaz de causar casos esporádicos e surtos de listeriose, uma doença invasiva severa. A proteína internalina A (InlA), codificada pelo gene *inlA*, tem um papel fundamental no processo de infeção, sendo necessária e suficiente para que *L. monocytogenes* ultrapasse a barreira intestinal e estabeleça uma infeção sistémica. Estudos sobre *inlA* de *L. monocytogenes* mostram que mutações que levam a codões stop prematuros ocorrem naturalmente e estão associadas a isolados com virulência atenuada. Foi sequenciado o gene *inlA* de onze isolados alimentares e onze isolados clínicos de *L. monocytogenes* recolhidos em Portugal com o objetivo de procurar mutações que levam ao aparecimento de codões stop prematuros, levando assim à produção de InlA truncada. Também foram testadas as eficiências de invasão de *L. monocytogenes* em linhas celulares Caco-2. Foram encontradas três tipos de mutações já descritas (6, 11 e 12), codificando uma proteína truncada com 492, 576 e 685 aminoácidos. Os codões stop prematuros foram identificados em quatro isolados alimentares e num isolado clínico. Os resultados obtidos indicam que os isolados com codão stop prematuro no gene *inlA* revelam uma eficiência de invasão atenuada em células Caco-2.

keywords

Caco-2 invasion assay, Internalin A gene, *Listeria monocytogenes*, Premature Stop Codons.

abstract

Listeria monocytogenes is a food-borne pathogen capable of causing listeriosis, a severe invasive disease and responsible for some outbreaks that occur occasionally. Internalin A protein (InlA) encoded by *inlA* gene has a key role in the infection mechanism, being necessary for *L. monocytogenes* to cross the intestinal barrier and to establish a systemic infection. Studies on *inlA* in *L. monocytogenes* have shown that mutations leading to premature stop codons (PMSC) occur naturally and are associated with attenuated virulence of this bacterium. *inlA* from eleven food and eleven clinical isolates of *L. monocytogenes* from Portugal, were sequenced to find mutations carrying PMSC leading to production of a truncated and secreted InlA. The invasion efficiencies of these strains of *L. monocytogenes* of the Caco-2 cell line were also tested. It was found three mutation types, previously described (6, 11 and 12), leading to a predict truncated InlA of 492, 576 and 685 amino acids. The PMSCs were detected in four food and in one clinical isolates. The results show a reduced invasiveness, in Caco-2 cell line, of isolates with PMSCs in *inlA*.

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List of abbreviations

aa	Amino acids
BHI	Brain Heart Infusion
bp	Base pair
CSF	Cerebro-spinal fluid
CFU	Colony Forming Units
inl	Internalin gene
Inl	Internalin protein
L.	*Listeria*
LRR	Leucine-rich repeat
nt	Nucleotide
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PMSC(s)	Premature Stop Codon(s)
UK	United Kingdom
USA	United States of America

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1 Introduction

1.1 *Listeria monocytogenes*

Listeria monocytogenes is a food-borne pathogen belonging to genus *Listeria*, motile, nonspore forming and non-encapsulated Gram positive bacteria, with low G+C DNA content as in other genera such as *Staphylococcus*, *Streptococcus*, *Lactobacillus* and *Bronchothrix* of the sub-branch *Clostridium* [2]. The *Listeria* genus comprises seven other species, namely: *L. seeligeri*, *L. innocua*, *L. welshimeri*, *L. grayi* [65], *L. marthii* [21], *L. rocourtii* [31] and *L. ivanovii* [65].

Listeria monocytogenes, was first discribed in 1926, at Cambridge, England, as the etiological agent of a septicemic disease affecting rabbits and guinea pigs characterized by peripheral monocytosis [1], by E.G.D Murray, R. A. Webb and M. B. R. Swann (1926) who named it *Bacterium monocytogenes*. Only in 1939 Pirie gave it the present name, *Listeria monocytogenes*, that was recognized by the International Committee on Systematic Bacteriology. In 1929, *L. monocytogenes* was isolated from infected humans by Nyfeldt, in Denmark [22]; the first *L. monocytogenes* human outbreak occurred in 1983 in Canada and the considerable clinical and microbiological interest in *L. monocytogenes* began after that [14, 22].

While *L. monocytogenes* is considered a major intracellular bacterial food pathogen, capable of causing serious disease, it is also well adapted to survive in soil and decaying vegetation as a saprophyte [23]. This bacterium grows between 0 and 45 ºC, with an optimum temperature range between 30 and 37 ºC; in a wide pH range, 4 to 9.6, and tolerates high salt concentrations, up to 10% [13]. Thus it can be found in several environments such as soil, water, food (eg. vegetables, fermented sausages, seafood) and as an animal pathogen [45], being a ubiquitous microorganism.

To distinguish *L. monocytogenes* from other species of *Listeria* spp. some biochemical tests can be performed, e.g using the comercial kit API based on the fermentation of sugars and enzymes activity and by combinations of the following tests: hemolysis, acid production from D-xylose, L-rhamnose, alpha methyl-D-mannoside, and mannitol [55].

Listeria spp. are catalase positive, and indole and oxidase negative, can hydrolyse aesculin but not urea and they vary in their ability to haemolyse horse or sheep red blood cells. *Listeria monocytogenes* is β-haemolytic positive as are *L.*

ivanovii and *L. seeligeri*, but it can be distinguished from these two species because it ferments rhamnose and does not ferment xylose unlike *L. ivanovii* and *L. seeligeri* [36]. Growth in PALCAM *Listeria* Agar, a selective and differential agar medium, is another way for isolation and enumeration of *Listeria* spp. and to detect *L. monocytogenes* from food and clinical samples [11]. ALOA (Agar Listeria Ottaviani & Agosti) can also be used for isolation of *Listeria* spp. from food samples and for presumptive identification of *L. monocytogenes* [38]. Identification of the species can be performed by molecular methods based on genotype as, e.g., 16S rRNA nucleic acid amplification [2, 36].

Thirteen serotypes have been described for *L. monocytogenes* based on surface proteins as somatic (O) and flagellar (H) antigens, and its agglutination by specific antisera. Serotypes 4b, 1/2b, and 1/2a account for more than 95% of human listeriosis cases [30, 61]**.** Serotype 4b is overrepresented among clinical isolates relative to food isolates and is associated with more severe clinical presentation and higher mortality rates [61]. Strains of this serotype contribute significantly to sporadic listeriosis and are responsible for most listeriosis outbreaks, including three previously defined epidemic clones (ECs) responsible for multiple outbreaks worldwide [29, 30].

Molecular subtyping studies consistently showed that *L. monocytogenes* isolates cluster into four divergent genetic lineages, represented on table 1, namely: lineages I, II, III and IV. Lineage I includes serotypes 1/2b, 3b, 3c, and 4b; lineage II includes serotypes 1/2a, 1/2c and 3a; lineage III includes at least three different lineages (lineage IIIA, IIIB and IIIC), which all contain serotype 4a, 4c, and atypical serotype 4b isolates. Several recent phylogenetic studies have demonstrated that isolates classified as subgroup IIIB represent a distinct fourth lineage of *L. monocytogenes* [36, 47, 52]. While Lineage I isolates are significantly overrepresented among human listeriosis cases (in particular serotype 4b strains) [24, 27], Lineage II isolates have a higher prevalence among environmental samples, foods, and animal listeriosis cases [41, 57], and are underrepresented among clinical isolates [27]. Lineage III is mainly associated with listeriosis in animals [36].

The first method used for differentiating *L. monocytogenes* was serotyping; today other methods have been used, such as ribotyping, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

Table 1 Summary of *L. monocytogenes* lineages.

Lineage	Initial identification	Serotypes	Genetic characteristics	Distribution
I	Piffaretti *et al*. [48]	1/2b, 3b, 3c, 4b	Lowest diversity among the lineages; lowest levels of recombination among the lineages.	Commonly isolated from various sources; over-represented among human isolates.
II	Piffaretti *et al*. [48]	1/2a, 1/2c, 3a	Most diverse, highest recombination levels.	Commonly isolated from various sources; over-represented among food-related as well as natural environments.
III	Rasmussen *et al*. [51]	4a, 4b, 4c	Very diverse; recombination levels between those for lineage I and lineage II.	Most isolates obtained from ruminants.
IV	First reported as IIIb by Roberts *et al.* [53] First reported as IV by Ward *et al*. [66]	4a, 4b, 4c	Few isolates analyzed to date.	Most isolates obtained from ruminants.

Adapted from Orsi *et al*. [21]

1.2 Listeriosis

Listeria monocytogenes can cause the serious infection, termed listeriosis. In developed countries, the globalization of the food industry and the increase in consumption of ready-to-eat, particularly chilled foods, has increased listeriosis outbreaks in the last 30 years [1].

Listeria monocytogenes has the ability to induce its own entry into host cells, usually mammalian cells (human and animal), such as macrophages, epithelial cells and endothelial cells of the gastrointestinal tract [9, 14] after the ingestion of contaminated food. It has been estimated that food-borne infections of listeriosis result in 90% of hospitalization and 20 to 30% of mortality of patients [8, 30, 69].

The majority of cases are found in immunosupressed patients (e.g. people with cancer, HIV infection, diabetes mellitus, heart problems, alcoholic individuals with cirrhosis, renal failure with dialysis), the elderly and pregnant women, as well as neonates [1, 14, 37]. *Listeria monocytogenes* infection can result in bacterial meningitis, rhombencephalitis, sepsis, endocarditis, pneumonia, hepatitis, peritonitis, osteomyelitis and gastroenteritis, the most common feature, in adults. [1]. There are subclinical cases, described in the USA, Italy and Scandavia, of listeriosis with symptoms like mild influenza [3, 37].

Listeriosis during pregnancy can cause amnionitis, preterm labor, spontaneous abortion, stillbirth or early-onset infection of the neonate. The early-onset results from intrauterine infection and it presentation is most often sepsis and sometimes respiratory distress [59]. The late-onset, 7-20 days after childbirth, is very similar to neonatal meningitis resulting in ≈ 10% of mortality [1].

Listeriosis can be treated with bacteriostatic concentrations of ampicillin or vancomycin combined with an aminoglycoside (normally gentamycin) or trimethoprim-sulfamethoxazole with rifampicin during three weeks. Usually, *L. monocytogenes* is resistant to cephalosporins [1].

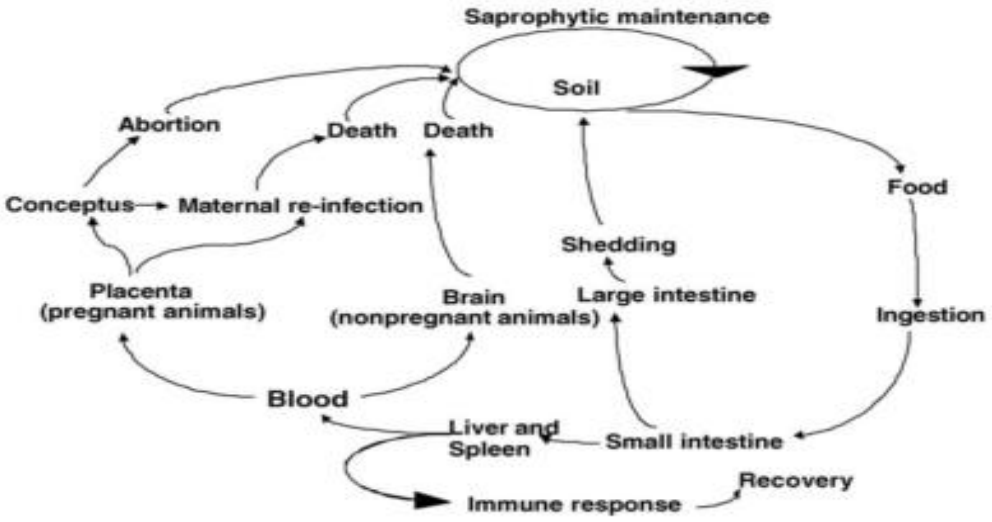

Figure 1 Saprophytic and infectious cycles of *L. monocytogenes*. In Orndorff *et al*. [44].

Figure 1 demonstrates the saprophytic and infectious cycles of *L. monocytogenes* and its ability to infect organs such as brain, liver, placenta and bloodstream [18, 44]. The infection by *L. monocytogenes* mainly occurs by the ingestion of contaminated food [8] and, although the acid pH of stomach, some *L. monocytogenes* passthrough to the small intestine, the first site where invasion occurs [37]. From there *L. monocytogenes* can spread from cell to cell without being in the extracellular environment, thus escaping from the human T-cell immune system, and invading other tissues and organs [18]. *Listeria monocytogenes* cell infection can be divided in four main steps (Figure 2) associated with different virulence factors [9, 44].

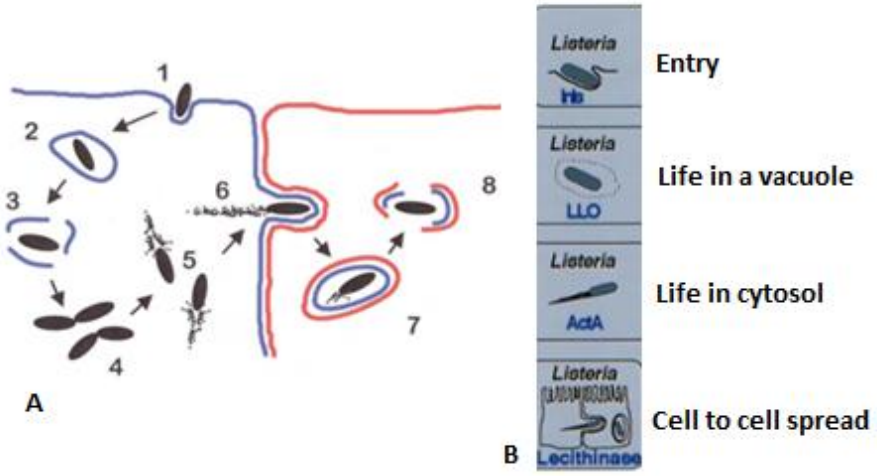

Figure 2 A: *Listeria* invasion process. 1 entry into host cell – zipper mechanism; 2 and 3 survival in and lysis of phagocytic vacuole by acidification; 4 duplication in 1 hour; 5 motility based on actin filaments polymerization; 6 cell-to-cell spread by double membrane vacuole formation; 7 and 8 survival and lysis of double membrane phagosomes. Adapted from Vázquez-Boland *et al.* [65]. B: Virulence factors required for *Listeria* invasion. Adapted from Cossart and Lecuit [10].

To invade, *L. monocytogenes* needs to adhere to the surface of host cells through the family of internalin surface proteins (InlA and InlB mainly) that are reconized by a specific host surface protein. Host cells recognize InlA through E-cadherin [9] and InlB through the hepatocyte growth factor (HGF) receptor Met [4]. Once inside the host cell, *L. monocytogenes* has to survive in a phagocytic vacuole and for that it uses two more virulence factors: listeriolysin O (LLO) and phospholipase C (PLC). The listeriolysin O is a cytolysin that forms a pore; phospholipase C (PI and PC) are responsible, at the same time, for lysing the vacuole formed around *L. monocytogenes*. The last step involved in the *L. monocytogenes* infection process, is its transfer into neighbouring cells that occurs in the same way of its entrance into the host cell, ie, with LLO and PLC action to lyse the second vacuole previously formed [9, 44, 65].

1.3 Internalin A

Internalin A protein (InlA) has been shown to be the most important factor for host-cell invasion because it acts as both adhesins and integrins and Lecuit *et al*. [35]. suggested that InlA is the key virulence factor in oral infections. Although InlA and InlB have been recognized as the most important *L. monocytogenes* cell-surface proteins responsible for promoting host cell invasion, 24 more internalin proteins have been described [5, 33].

InlB belongs to the second group of surface proteins in Gram-positive bacteria that bind to the cell wall by non-covalent interactions. This protein has a signal peptide, at leucine-rich repeat (LRRs) domains and a C-terminal domain with three highly conserved tandem repeats of 80 amino acids, and the GW modules (Gly-Trp), necessary to anchor InlB to the bacterial surface [4, 6].

InlA protein is the first virulence factor identified in *L. monocytogenes* allowing the entry of the bacterium into non-phagocytic host cells, this process is called internalization. The interaction with the host cell occurs by the linkage of InlA to the human isoform of E-cadherin transmembranar protein, allowing *L. monocytogenes* to cross the intestinal and feto-placental barriers [32, 39, 46, 70]. E-cadherin is a cell adhesion protein of mammalian cells, necessary for the formation of cell-cell junctions of polarized epithelial cells and is connected to cytoskeletal proteins. The recognition of InlA occurs through Pro residue on the position 16 in a hydrophobic and uncharged loop of E-cadherin, suggesting that InlA-E-cadherin interaction is hydrophobic [4, 5, 58, 62].

InlA is an 800 aa surface protein (Figure 3) belonging to the LPXTG proteins [46] with a signal peptide in the amino-terminal, followed by a LRR domain (a tandem repeat of 20-22 aminoacids with a role in protein interactions and activation); the next region is an inter-repeat domain (IR) crucial for the interaction with E-cadherin and a β-repeat region. The last region, at the carboxyl-terminal, is the sorting signal which has the LPXTG motif (X is any aminoacid), a hydrophobic region with 20 aminoacids and a tail of positive residues. The carboxyl-region is fundamental for covalent attachment of InlA to the peptidoglycan of the Gram-positive cell wall, by the action of sortase A (SrtA) [4, 6, 19, 28, 33, 70].

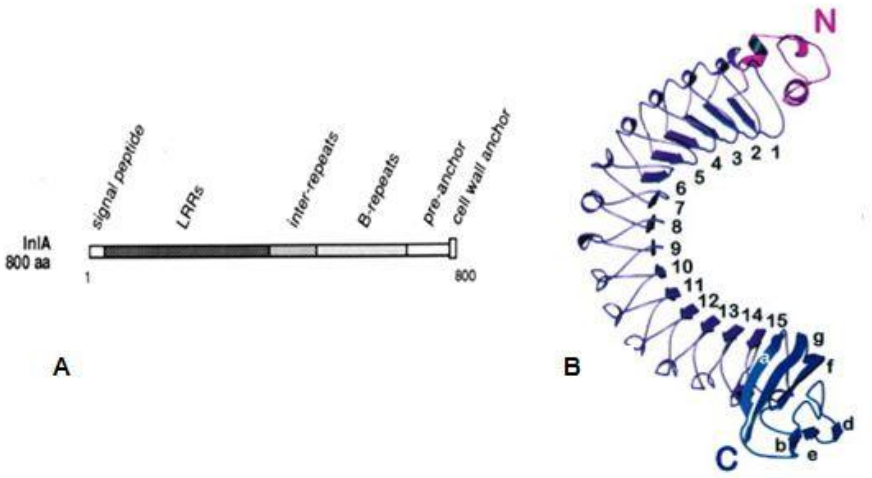

Figure 3 A: Schematic representation of InlA. Adapted from Lecuit *et al*. [33]. B: Uncomplexed InlA. Adapted from Schubert *et al*. [21]

Interesting data from Gaillard *et al*. [19] shows that InlA confers invasiveness to non-invasive Gram-positive bacteria, as, e.g., *L. innocua* by the introduction of a plasmid pGM4 with *inlA* into noninvasive *L. innocua* [19].

Jonquières *et al*. [28] compared the invasiveness of strain LO28 and strain EDGe, in Caco-2 cells, and concluded that strain LO28 presented a low internalization efficiency and the Western blotting analysis demonstrated a protein with lower molecular weight when compared with EDGe protein. These results indicated the ocurrence of frameshift mutations in *inlA* gene, resulting in premature stop codons (PMSC). Thus the expressed InlA protein is truncated or not secreted, if the PMSC is located at 5´end of the gene [40], and *L. monocytogenes* therefore cannot be detected by E-cadherin of the host cells [5, 28]. Figure 4 presents examples of truncated InlA and the incapacity to be recognized by the host cell. The same study showed that this is not a rare event and later studies confirmed these observations, emphasizing the idea of InlA as a key virulence factor in *L. monocytogenes* [5, 28, 41]. The truncated form of InlA appears more in food strains, while in clinical strains the full-length InlA usually occurs [5, 26, 40, 41]. These data prove the fundamental role of InlA in the crossing of the intestinal barrier in humans.

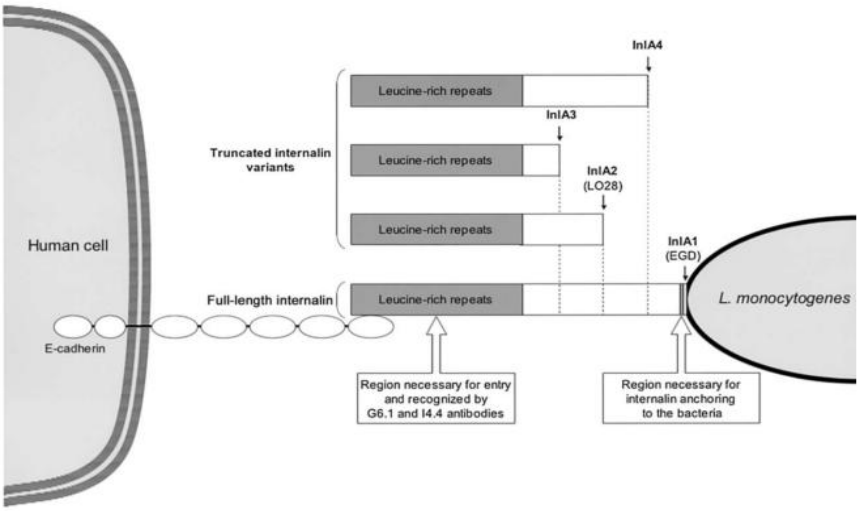

Figure 4 Interaction of InlA protein and E-cadherin and truncated forms of InlA in *L. monocytogenes*. InlA1: full-lengh internalin, capable of promoting entry of *L. monocytogenes* into host cells because it retains the carboxy-terminal motif for anchoring to the bacterial surface. InlA2, InlA3 and InlA4: variants of truncated internalins. *In* Jacquet *et al*. [26]

So far, 18 types of *inlA* mutations leading to PMSCs that result in a truncated form of InlA incapable of anchoring to the bacteria cell wall, have been described (Figure 5) [63, 64]. Mutations leading to PMSC in *inlA* were found in isolates collected in several countries e.g. USA [41, 46], Portugal [15, 16], France [26, 28, 42] and Japan [25]. Usually, *inlA* from *L. monocytogenes* isolates are sequenced and compared with the *inlA* gene from the EDGe strain that secretes a full-length InlA; this methodology allows the detection of PMSC.

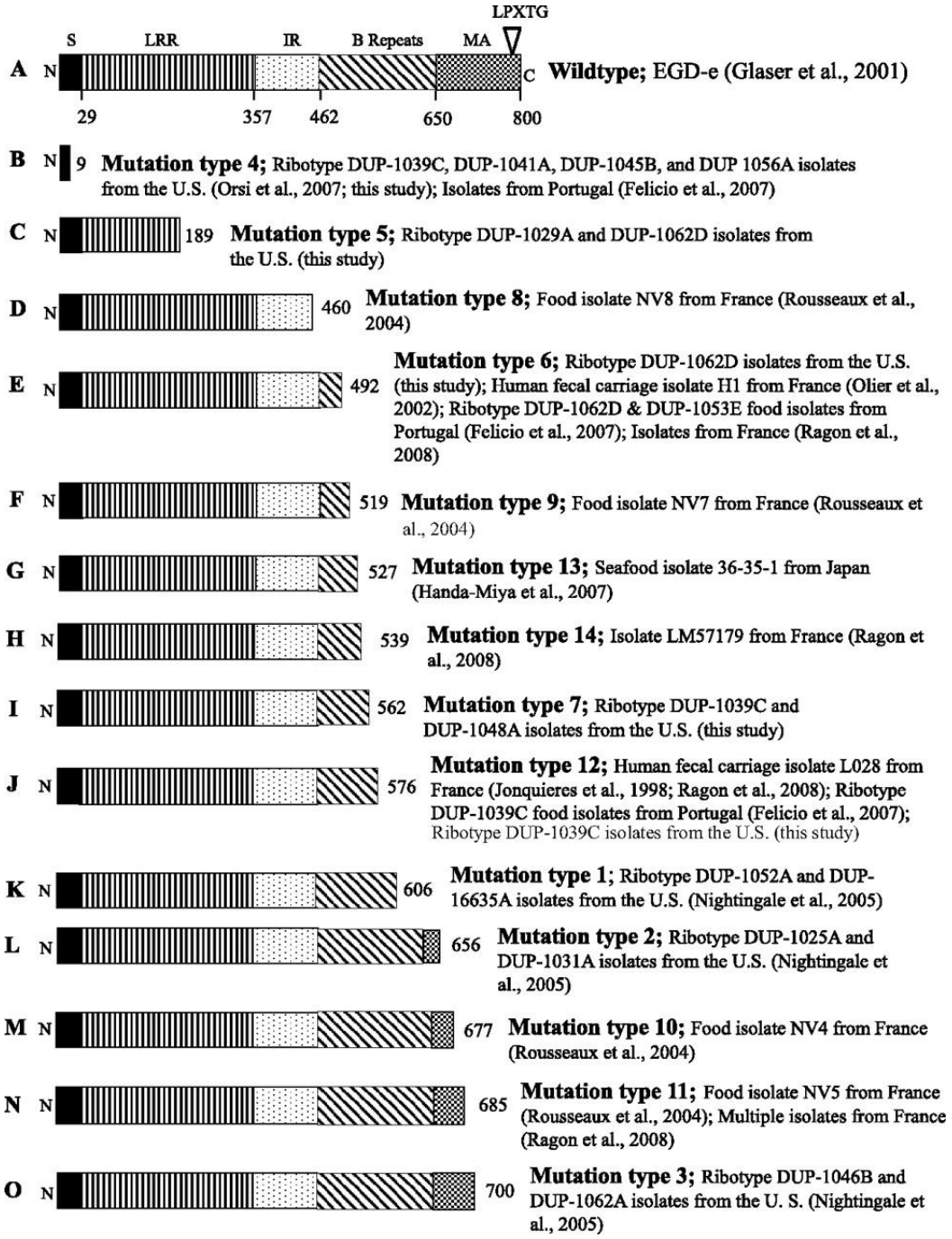

Figure 5 Representation of full-length InlA (A) and truncated InlA (B – O) with respective mutation type, described by several studies. N, N-terminal; S, signal sequence; LRR, leucine-rich repeat; IR, intergenic repeat; MA, membrane anchor; C, C-terminal end. Numbers at the right end for lines B to N represent the aa position of each respective PMSC. *In* Van Stelten *et al*. [64]

The first frameshift mutation (an adenine deletion at 1637 position) leads to a nonsense codon (TAA) in the position 1729 that encodes for a 63 kDa protein instead of an 88 kDa protein, was described by Jonquières *et al*. [28]. Ten more studies,

between the year 1998 and 2010, have found 17 mutations leading to PMSCs, described in detail on Table 2.

Table 2 PMSC mutations described since 1998 to 2010 in *inlA*.

PMSC mutation type[a]	Protein length (aa)	Mutant allelic type	Wild allelic type	Nucleotide position of PMSC mutation	Isolate type	Serotype	Country origin	Reference
1	606	A	T	1818	Clinical Food Environmental	1/2b	USA	Nightingale *et al*. [41] Van Stelten and Nightingale [63] Van Stelten *et al*. [64]
2	656	T	C	1966	Clinical Food Environmental	1/2b	USA	Nightingale *et al*. [41] Van Stelten and Nightingale [63] Van Stelten *et al.* [64]
3	700	G	C	2100	Clinical Food Environmental	1/2a	USA	Nightingale *et al*. [41] Van Stelten and Nightingale [63] Van Stelten *et al.* [64]
4	9	C	A	6 -12 Adenine residues	Clinical Food Environmental	1/2c or 3a	Portugal USA	Orsi *et al*. [46] Felício *et al*. [15] Van Stelten and Nightingale [63]
5	189	T	C	565	Clinical Food	[b]	USA	Van Stelten and Nightingale [63] Van Stelten *et al*. [64]
6	492	T	C	1474	Clinical Food	[b]	France Portugal USA	Olier et al. [43] Felício *et al*. [15] Van Stelten and Nightingale [63] Van Stelten *et al*. [64] Ragon *et al*. [49]
7	562	T	C	1684	[b]	[b]	USA	Van Stelten and Nightingale [63]
8	460	-	G	1539 deletion	Food	1/2a	France	Rousseaux *et al*. [56]

9	519	-	G	1539 deletion	Food	1/2c	France	Rousseaux *et al*. [56]
10	677	T	-	1901 insertion	Food	1/2a	France	Rousseaux *et al*. [56]
11	685	A	G	2054	Food	1/2c	France	Rousseuax *et al*. [56] Ragon *et al*. [49]
12	576	-	A	1637	Clinical Food	1/2c or 3c	France Portugal USA	Jonquières *et al*. [28] Rangon *et al*. [49] Felício *et al*. [15] Van Stelten and Nightingale [63] Van Stelten *et al*. [64]
13	527	T	A	1578	Food	1/2a	Japan	Handa-Miya *et al*. [25]
14	539	T	C	1615	[b]	[b]	France	Ragon *et al*. [49]
15	77	T	C	b	Food	1/2a	USA	Ward *et al*. [67]
16	170	T	G	b	Food	1/2b	USA	Ward *et al*. [67]
17	253	A	T	b	Food	1/2b	USA	Ward *et al*. [67]
18	404	-	T	1165 (deletion)	Clinical	4b	USA	Ward *et al*. [67]

[a] According to Van Stelten *et al*. [64]; [b] Information not available

Van Stelten *et al.* [64] hypothesised that isolates with mutations type 1, 3 and 4 have been accumulated at the population level, since these mutations represent 90% of the *inlA* PMSC mutations observed in USA isolates (mostly mutation type 3). Futhermore, *inlA* mutations leading to PMSCs seem to be a common event in food isolates and for the most part, the described mutation types are exclusively in isolates from each respective country where those mutations were originally described [64].

Listeria monocytogenes isolates with PMSC mutation leading to a truncated form of InlA, usually have reduced invasion efficiency in the human colorectal adenocarcinoma epithelial cell line - Caco-2 cells. So the invasion assays with Caco-2 are a strong tool to predict the presence of *inlA* mutations [40, 63, 66]. Gaillard *et al*. [20] used the Caco-2 cell line to investigate the ability of *L. monocytogenes* to enter into intestinal epithelial cells, through the specific interaction with E-cadherin [35, 60], as it is the only cell line similar to differentiated enterocytes [20] and *L. monocytogenes* entry depends on the InlA pathway [7]. In several studies Caco-2 invasion assays have been selected to demonstrate reduced invasion efficiency of *L. monocytogenes* carrying a PMSC mutation and to correlate this data with reduced human virulence of some strains of *L. monocytogenes* [15, 40-42, 63]. Nightingale *et al*. [40] demonstrated the interesting result that human exposure to attenuated virulence of food isolates carrying PMSC mutations in inlA, may confer protection against virulent isolates (with full length InlA), thus starting a new approach to listeriosis prevention [40, 64].

1.4 Aim of thesis

The surface protein InlA, encoded by *inlA*, has been associated with the invasion of human intestinal epithelial cells by *L. monocytogenes* that express certain isoforms of E-cadherin, making the interaction between InlA and E-cadherin a critical first step for crossing the intestinal barrier during the initial stages of a *L. monocytogenes* infection. Isolates harboring PMSC mutation in *inlA*, resulting in truncated forms of InlA, present virulence-attenuated phenotypes and impaired invasiveness of human intestinal epithelial cells in animal models. Increasing evidence suggests that *inlA* PMSC mutations are frequent in strains isolated from foods, but rare among clinical isolates. Limited information is available on the prevalence of such mutations among isolates collected from Portuguese samples. Such information would be critically

needed for adequate assessments of the foodborne illness burden associated with *L. monocytogenes* strains circulating in the country.

The aim of this study was to evaluate the prevalence of PMSCs in *inlA* of *L. monocytogenes* strains collected from food and clinical samples in Portugal. Characterization of the strains was accomplished (i) phenotypically by Caco-2 invasion efficiency assays and (ii) genetically by *inlA* sequencing.

2 Materials and methods

2.1 *Listeria monocytogenes* isolates

For this study a set of 22 bacterial isolates from food and clinical origin collected in Portugal between 2001 and 2011 were selected from the *L. monocytogenes* collection of *Escola Superior de Biotecnologia-Universidade Católica Portuguesa.* Some isolates had previously been characterized by Pulsed Field Gel Electrophoresis (PFGE) and geno-serotyping by multiplex-PCR as previously described by Doumith *et al.* [12]. Food and clinical isolates previously studied by Ramalheira *et al*. [50], for their capacity to survive through simulated gastrointestinal tract conditions, representing several PFGE types and serotypes, were selected to represent the diversity of *L. monocytogenes* associated with different food sources and clinical cases. Furthermore, two clinical (2562 and 2666) and one food (2652) isolates related to the Portuguese outbreak of listeriosis that occurred in the Region of Lisboa and Vale do Tejo in 2010, were selected. Isolates FSL F8-146 and FSL F8-144 were used as positive and negative controls, respectivelly. Information on the selected strains of *L. monocytogenes* are described on Table 3.

Table 3 *L. monocytogenes* strains characterization.

[a]*L. monocytogenes* isolate	Origin		Serogroup	[a]PFGE pulsotype	Date
747	Food	Cheese	1/2b	0040	2003
842	Food	Swab sample	1/2c	0208	2003
854	Food	Lettuce salad	4b	----	2003
903	Food	Roast chicken	4b	----	2004
925/3	Food	Goat cheese	1/2b	0016	2004
930/1	Food	Goat milk	1/2b	----	2004
994/1	Food	Goat milk	1/2a	0005	2004
1044	Food	Meal	4b	----	2004
1216	Food	Raw chicken	1/2b	0278	2004
1305	Food	Non-fermented sausage	1/2c	0206	2005
2652	Food	Goat and cow	4b	0101	2011

		fresh cheese			
1001	Clinical	Blood	4b	0019	2004
1547	Clinical	Blood	1/2b	0008	2001
1761	Clinical	CSF catheter	1/2b	0032	2005
1891	Clinical	CSF	1/2b	0048	2006
2065	Clinical	Blood	1/2b	0037	2007
2074	Clinical	Blood	4b	0072	2007
2086	Clinical	Blood	1/2a	0036	2007
2092	Clinical	Blood	4b	0065	2007
2103	Clinical	Blood	1/2a	0009	2007
2562	Clinical	Blood	4b	0101	2010
2666	Clinical	Blood	4b	0070	2010
FSL F8-144	Control	n/a	1/2a	356	2004
FSL F8-146	Control	n/a	1/2b	346	2004

[a]Codes in *L. monocytogenes* collection of Escola Superior de Biotecnologia-Universidade Católica Portuguesa. n/a – not applicable

2.2 DNA extraction

For each isolate, one colony grown on Brain Heart Infusion (BHI) (Lab M Limited, Lancashire, UK) Agar No 1 Bacteriological (Lab M Limited, Lancashire, UK) (37 ºC, overnight) was cultured in BHI broth and incubated at 37 ºC, overnight. A 250 µL aliquot of bacterial cells was pelleted (10,000 g) for 10 min and re-suspended in 95 µL of 1x PCR buffer (Fermentas, Mundolsheim, France) and 4 µL of a 50 mg/mL lysozyme solution (Sigma, Steinheim, Germany). After incubation at room temperature (RT) for 15 min, 1 µL of 20 mg/mL proteinase K (Eurobio GEXPRK01-B5, Scandinavia) solution was added and incubated at 58 ºC for 60 min. Enzymes were heat inactivated at 95 ºC, for 10 min, and the DNA stored at -20 ºC.

2.3 *internalin A* Sequencing

Listeria monocytogenes isolates were screened for the presence of PMSCs in *inlA* using the pairs of primers described in Table 4. *inlA* gene was amplified by PCR using four pairs of primers that cover the whole *inlA* ORF.

For each isolate, a 25 µL PCR reaction mixture was prepared containing: 1x KAPA HiFi Fidelity Buffer containing 2.0 mM Mg^{2+} (KAPA Biosystems, Woburn, USA), 0.3 mM of each deoxynucleotides triphosphate (Bioron, Ludwigshafen, Germany), 0.3 µM of each primer (Stabvida, Lisboa, Portugal) and 0.02 U/µL of KAPA HiFi DNA Polymerase (KAPA Biosystems), and 0.5 µL of DNA extract.

For the primers *inlA* proF/*inlA* proR, *inlA* F1/*inlA* S1R and *inlA* F2/*inlA* S2R the cycle used was: 3 min at 95 ºC; 30 cycles of 20 sec at 98 ºC, 15 sec at 62 ºC and 15 sec at 72 ºC; and a final cycle of 5 min at 72 ºC. The primer *inlA* seqF/*inlA* R has the same cycle except the annealing temperature was 67 ºC. The PCR reaction was performed in DNA Engine Peltier Thermal Cycler Chromo 4 (Bio-Rad, Hercules, USA).

The electrophoresis was performed with 5 µL of PCR product and 3 µL of Sample Loading Buffer (Bioline, UK) in a 1% SeaKem ® LE Agarose gel (Lonza, Rockland, USA) containing 1000x Midori Green Advanced DNA Stain (Nippon Genetics Europe GmbH, Düren, Germany), for 45 min at 95 V. Gels were photographed under UV transillumination (GelDoc 2000, Bio-Rad).

The PCR products were purified using the EzWay PCR Clean-Up Kit (Komabiotech, Seoul, Korea) and the DNA sequencing was performed by Macrogen Inc., Korea, using the same primers used for PCR of *inlA* amplification. The analysis of the sequences was done on Geneious 4.8.2 program and on Mega 5.05.

Table 4 Primers for *inlA* PCR and sequencing

Primer	Sequence (5' – 3')[a]	Amplicon size
***inlA* proF** ***inlA* proR**	For: TTT TAA AAG GTG GAA TGA CA Rev: GAA GCG TTG TAA CTT GGT CTA	500 bp
***inlA* F1** ***inlA* S1R**	For: CAG GCA GCT ACA ATT ACA CA Rev: GGA CTG ATG TTA CTT ATT TGG T	800 bp
***inlA* F2** ***inlA* S2R**	For: AAG ATA TAG GCA CAT TGG CGA GTT Rev: CGT ACT GAA ATY CCA KTT AGT TCC	800 bp
***inlA* seq F** ***inlA* R**	For: GTG GAC GGC AAA GAA ACA AC Rev: ATA TAG TCC GAA AAC CAC ATC T	900 bp

[a] For, forward; Rev, reverse

2.4 Caco-2 cells invasion assays

Caco-2 (tumor-derived human colorectal epithelial cell line) cells invasion assays were performed to determine the invasion efficiency of *L. monocytogenes* as it requires InlA protein. A reduction in invasion efficiency could be explained by *inlA* mutations that code for a truncated protein [40].

The Caco-2 cells were grown in a T75 flask using Eagle´s minimal essential medium (EMEM) (Lonza, Verviers, Belgium) containing 20% fetal bovine serum (FBS) (Lonza), 1% sodium pyruvate (Lonza) and 1% non-essential aa (Lonza). For the invasion assays, $5.0x10^4$ Caco-2 cells per well in 24-well plates were used, previously grown in EMEM with FBS, sodium pyruvate and non-essential aa, and were incubated for 48 h at 37 ºC with 7% (v/v) CO_2 atmosphere.

Listeria monocytogenes strains were grown in BHI for 16 h at 37 ºC without shaking, and approximately $2x10^7$ CFU/mL were inoculated into Caco-2 monolayers and allowed an infection time of 60 min at 37 ºC with 7% (v/v) CO_2, in triplicate wells. The exact inoculum was determined by plating on BHI agar. Following the infection time, the medium was aspirated and gentamicin solution 20% (Lonza) was added and incubated for 90 min at 37 ºC with 7% (v/v) CO_2, to kill remaining extracellular bacteria. After the gentamicin action, the medium was aspirated and cells washed with PBS. Post-infection, Caco-2 cells were lysed with 1 mL of Triton solution 0.2% (Sigma) for 10 min at room temperature and collected with vigorous pippeting. Intracellular *L. monocytogenes* cells were enumerated on BHI plates, which were subsequently incubated at 37 ºC for 24 h. Two control strains were included in each invasion assay; FSL F8-146 as a 100% invasion efficiency and FSL F8-144 as a 2% low invasion efficiency [16, 40].

3 Results

3.1 *inlA* sequencing of *L. monocytogenes* isolates

Sequencing analysis of *inlA* of the 22 *L. monocytogenes* isolates described in Table 5 shows that isolates 854, 903, 925/3, 930/1, 994/1, 1044, 1001, 1547, 1891, 2065, 2074, 2086, 2092, 2103, 2562, 2652 and 2666 do not have mutations leading to a truncated form of InlA.

Listeria monocytogenes food isolate 842 shows a mutation type 12, leading to PMSC in the nucleotide 1729, with a deletion of an adenine (A) in the position 1637, the predicted InlA protein of this isolate has 577 aa. Isolate 1216 and 1761, food and clinical isolates respectively, show the same mutation type 6, leading to PMSCs in the nucleotide 1474 after a substitution of one citosine for one thymine (T) in the position 1474, the predicted InlA protein has 492 aa. Only one clinical isolate (1761) shows a mutation in *inlA* leading to a truncated form of InlA. The food isolates 747 and 1305 show a mutation type 11, leading to PMSCs in the nucleotide 2053, with a substitution of one guanine (G) for an adenine in the position 2054, the predicted protein has 685 aa.

Table 5 *inlA* mutations leading to PMSC found in *L. monocytogenes* isolates.

L. monocytogenes isolate	*inlA* type of mutation	Mutant allelic type	Wild allelic type	Nucleotide position of PMSC mutation	Length of predicted protein (aa)
747	Mutation 11	A	G	2054	685
842	Mutation 12	n/a	A	1729	576
854	No mutation	n/a	n/a	n/a	800
903	No mutation	n/a	n/a	n/a	800
925/3	No mutation	n/a	n/a	n/a	800
930/1	No mutation	n/a	n/a	n/a	800
994/1	No mutation	n/a	n/a	n/a	800
1044	No mutation	n/a	n/a	n/a	800

1216	Mutation 6	T	C	1474	492
1305	Mutation 11	A	G	2054	685
2652	No mutation	n/a	n/a	n/a	800
1001	No mutation	n/a	n/a	n/a	800
1547	No mutation	n/a	n/a	n/a	800
1761	Mutation 6	T	C	1474	492
1891	No mutation	n/a	n/a	n/a	800
2065	No mutation	n/a	n/a	n/a	800
2074	No mutation	n/a	n/a	n/a	800
2086	No mutation	n/a	n/a	n/a	800
2092	No mutation	n/a	n/a	n/a	800
2103	No mutation	n/a	n/a	n/a	800
2562	No mutation	n/a	n/a	n/a	800
2666	No mutation	n/a	n/a	n/a	800
FSL F8-144	Mutation 4	C	A	12	9
FSL F8-146	No mutation	n/a	n/a	n/a	800

n/a – not applicable

3.2 Caco-2 cells invasion capability of *L. monocytogenes* isolates

The 22 *L. monocytogenes* isolates were characterized for invasion efficiency in Caco-2 cells (figure 6). FSL F8-146 was used as a positive control, defined with 100% invasion efficiency [16].

Figure 6 shows that clinical isolates 1891, 2086, 2092, 2562, 2065, 2666 and 2074 and food isolates 854, 2652 and 903 have high invasion efficiencies compared to the positive control (FSL F8-146). The food isolates 1216, 842, 1305, 1044, 994/1, 925/3, 930/1 and 747 and the clinical isolates 1547, 1761, 2103 and 1001 demonstrate reduced invasion efficiency compared with the positive control. The average invasion efficiencies for these isolates ranged from 0.9 to 65.2%, relative to the positive control. The maximum efficiency value observed was 739.4%, for the isolate 2074.

Invasion assays of isolates 1001, 1891, 2086, 903 and 2666 need to be repeated as high standart deviations were observed.

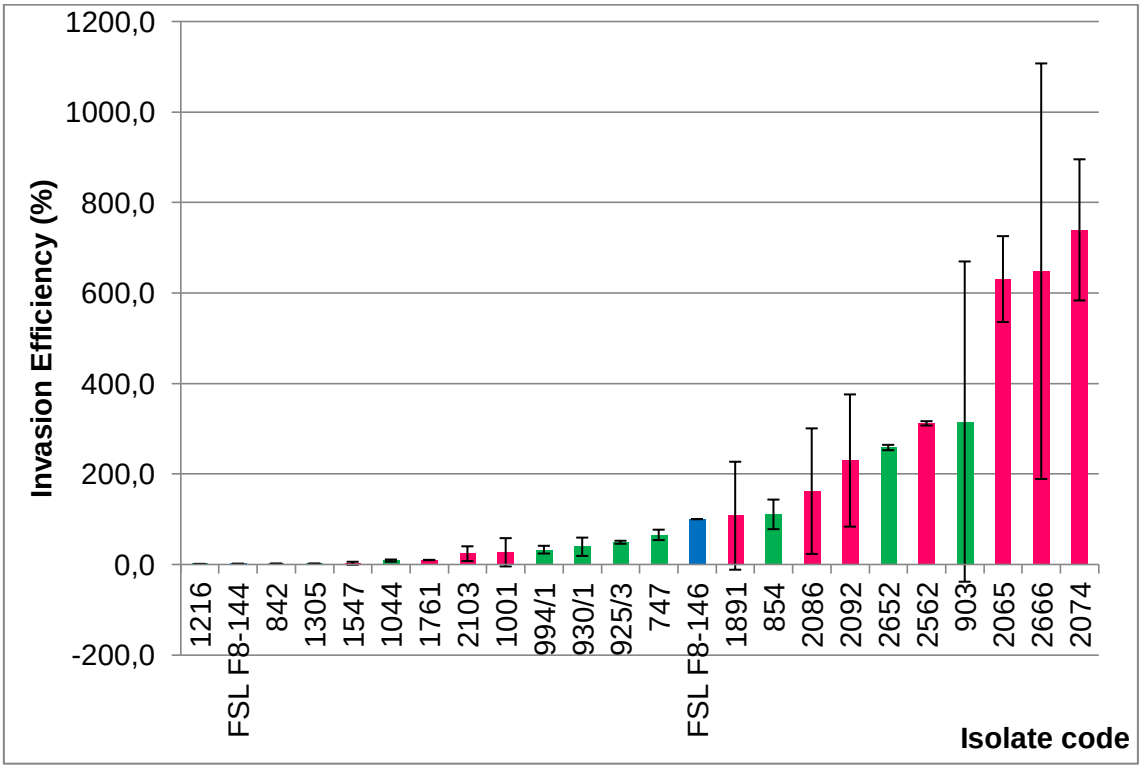

Figure 6. Caco-2 cell invasion efficiencies for 22 *L. monocytogenes* isolates. Values represent average invasion efficiencies (normalized to that of the control strain (FSL F8-146) for 2 - 3 independent replicates; error bars indicates ranges. FSL F8-144 isolate was included as an invasion attenuated control. Clinical isolates Food isolates Controls

4 Discussion

Twenty two Portuguese isolates of *L. monocytogenes* (food and clinical) were screend for the presence of PMSC mutations in *inlA* and also invasion assays were performed on Caco-2 cells to tentativelly relate the mutations with attenuated invasion phenotypes. An invasion efficiency of 100% was defined for the isolate FSL F8-146, based on results from Ferreira *et al.* [17] (positive control) as it does not show PMSC in *inlA*, encoding a predicted full-length InlA. All the other invasion efficiencies are values normalized to that of the FSL F8-146. It is important to point out that invasion efficiencies above 100% may not represent high virulence of the isolates as 100% invasion efficiency for strain FSL F8-146 is only a reference value.

All the three mutation types found (6, 11 and 12) in five isolates were previously described. The remaining seventeen isolates tested did not show PMSC in *inlA*.

Isolates 1761 and 1216 presented the mutation type 6. This mutation was first described by Olier *et al*. [43] in France, in an isolate from a healthy pregnant carrier. Five more research groups from France [49], Portugal [15] and USA [63, 64] described this mutation in food and clinical isolates. In this study, the mutation type 6 was found in a 1/2b clinical isolate (1761), the only clinical isolate that shows a mutation in *inlA* leading to a truncated form of InlA, and in a 1/2b food isolate (1216). Both isolates 1761 and 1216, showed reduced invasion efficiency in Caco-2 cells (9.5% in the case of strain 1761; 0.9% in the case of strain 1216), compared to the invasion efficiency of the positive control FSL F8-146.

Mutation type 11 was found in food isolates 747 and 1305, serotypes 1/2b and 1/2c, respectively. Rousseaux *et al.* [56] and Ragon *et al.* [49] had described the same mutation leading to a 685 aa. InlA in food isolates from France, belonging to serotype 1/2c, the same as isolate 1305, but we also found this mutation type in a 1/2b isolate (747). As predicted, these two food isolates showed reduced invasion efficiency in Caco-2 cells, with values 65.2% and 1.4% for 747 and 1305. Van Stelten *et al.* [64] noted that *inlA* PMSC mutations, except type 6 and 12, occur almost exclusively in isolates from countries where these mutations were originally described. However, in the present study it was found that mutation type 11, originally described from France, was also identified in two different food isolates from Portugal. So mutation type 11 is not exclusive from French isolates.

Food isolate 842 also showed a PMSC mutation in *inlA* leading to a truncated form of InlA and the evaluation of ability to invade Caco-2 cells demonstrated a reduced invasion efficiency (1.4%); both results are concordant, an isolate with PMSC leading to a truncated form of InlA shows attenuated invasion efficiency in Caco-2 cells. This mutation type (12) was the first mutation in *inlA* leading to a PMSC described in 1998 by Jonquières *et al.* [28] in France and, after that, was described also in France [49], in Portugal [15] and in USA [63, 64] in food and clinical isolates belonging to 1/2c and 3c serotypes.

These results confirm that isolates with PMSC mutations in *inlA*, which are predicted to lead to a truncated InlA, show attenuated invasiveness in Caco-2 cells. The three mutation types found, encode a truncated form of InlA with no cell wall anchor and, this way, *L. monocytogenes* is unable to adhere to the host cell by E-cadherin – InlA interaction. The reduced invasion phenotypes can be explained by three different mechanisms: (i) PMSC mutations in *inlA* leading to a truncated and dysfunctional InlA necessary for internalization of *L. monocytogenes* in human host cells, as demonstrated in Caco-2 cell line [34], (ii) reduced *inlA* transcript levels [54], and, (iii) reduced motility of *L. monocytogenes* [54]. Mechanisms (ii) and (iii) may explain the invasion efficiency results below FSL F8-146 invasion efficiency of 1547, 2103 and 1001 clinical isolates and 1044, 994/1, 930/1 and 925/3 food isolates, which do not show PMSC in *inlA*, analyzed by sequencing. Future studies will be necessary to understand the association between the three mechanisms to explain attenuated virulence of some food and clinical isolates.

PMSC mutations in *inlA* have been described as a common event in food isolates and a rare event in clinical isolates [64]; Jacquet *et al.* [26] demonstrated that 96% of the clinical strains have full-length InlA whereas only 65% of food strains show the full length protein. In this study we found PMSC in four food isolates and in one clinical isolate (1761) from a CSF catheter, belonging to lineage II (serotype 1/2a).

Epidemiologically, DNA sequencing of *inlA* and virulence phenotype studies show that, through consumption of contaminated foods, humans are frequently exposed to virulence-attenuated *L. monocytogenes* isolates carrying PMSC mutations in *inlA*. Because of heterogeneity in virulence among *L. monocytogenes* and the susceptibility of the host, an infectious dose is not defined. However it is known that ID_{50}s (dose required for systemic infection in 50% of the subjects) in guinea pigs is lower for an epidemic clone strain than for a isolate carrying a PMSC mutation in *inlA* [8]. Nightingale *et al.* [40] reported that oral exposure to *L. monocytogenes* with PMSC in *inlA* provides protection to subsequent exposure to virulent strains.

Listeria monocytogenes serotypes from isolates carrying PMSC in *inlA*, in this study, are 1/2b, belonging to lineage I, and 1/2a and 1/2c belonging to lineage II. Lineage I is over-represented among human cases and are associated with listeriosis outbreaks in USA and with sporadic cases in Northern Europe [45], in particular with serotype 4b. While lineage II are over-represented in food isolates [41, 57] and more than 30% show attenuated-invasion efficiency in Caco-2 cell line due to PMSC in *inlA* [45].

Listeriosis outbreaks are usually associated with milk and dairy products such as cheese and butter, and to RTE products [68]. In fact, the Portuguese outbreak isolate 2652 is from goat and cow fresh cheese and it belongs to lineage I (serotype 4b), over-represented in listeriosis outbreaks and it shows *inlA* leading to a full-length InlA, necessary to cause infection.

5 Conclusion

Listeria monocytogenes is a food-borne pathogen exhibiting considerable diversity in virulence-associated phenotypes dependent on serotypes and strain origin.

In this study it was possible to detect PMSCs in *inlA*, previous described, in food and clinical isolates from Portugal by *inlA* sequencing and characterize those isolates phenotypically through the efficiency of invasion in the Caco-2 cell line. In eleven food isolates, four of them (36.4%) display PMSCs in *inlA*, Only one clinical isolate (9.1%) shows PMSC, leading to a predicted InlA of 492 aa. So, PMSCs are prevalent in food isolates and under-represented among clinical isolates, despite the relatively low number of strains analyzed. Phenotypically, it was possible to relate the presence of PMSCs with invasion efficiencies below that of the positive control FSL F8-146. Isolates from the Portuguese outbreak reveal invasion efficiencies above 100% and do not show PMSCs in *inlA*, as expected.

InlA protein is necessary to cross the intestinal barrier, thus being a critical virulence factor to allow *L. monocytogenes* infection. When inlA shows PMSC leading to a truncated form of InlA, the isolate has a reduced invasion efficiency. It is necessary to understand the importance of attenuated-virulence strains with PMSC in inlA, mainly in foods, and how this could be explored to prevent risk of human disease.

Despite the important results obtained by Caco-2 invasion assays, it is necessary to study other *in vitro* and animal models to understand the pathophysiology of listeriosis *in vivo*.

6 References

1. Acheson, D. Foodborne Listeriosis. *Clinical Infectious Diseases* 2000, 770-775.
2. Allerberger, F. *Listeria*: growth, phenotypic differentiation and molecular microbiology. *Federation of European Microbiological Society Immunology & Medical Microbiology* 2003, 183-189.
3. Aureli, P., Fiorucci, G. C., Caroli, D., Marchiaro, G., Novara, O., Leone, L., Salmaso, S. An Outbreak of Febrile Gastroenteritis Associated with Corn Contaminated by *Listeria monocytogenes*. *New England Journal of Medicine* 2000, 1236-1241.
4. Bierne, H., Cossart, P. *Listeria monocytogenes* Surface Proteins: from Genome Predictions to Function. *Microbiololy and Molecular Biology Reviews* 2007, 377-397.
5. Bonazzi, M., Lecuit, M., Cossart, P. *Listeria monocytogenes* internalin and E-cadherin: from structure to pathogenesis. *Cellular Microbiology* 2009, 693-702.
6. Cabanes, D., Dehoux, P., Dussurget, O., Frangeul, L., Cossart, P. Surface proteins and the pathogenic potential of *Listeria monocytogenes*. *Trends In Microbiology* 2002, 238.
7. Cabanes, D., Sousa, S., Cebria, A., Lecuit, M., Garcia-del Portillo, F., Cossart, P. Gp96 is a receptor for a novel *Listeria monocytogenes* virulence factor, Vip, a surface protein. *Embo Journal* 2005, 2827-2838.
8. Chen, Y., Ross, W. H., Whiting, R. C., Van Stelten, A., Nightingale, K. K., Wiedmann, M., Scott, V. N. Variation in *Listeria monocytogenes* dose responses in relation to subtypes encoding a full-length or truncated internalin A. *Applied and Environmental Microbiology* 2011, 1171-1180.
9. Cossart, P. Molecular and cellular basis of the infection by *Listeria monocytogenes*: an overview. *International Journal of Medical Microbiology* 2002, 401-409.
10. Cossart, P., Lecuit, M. Interactions of *Listeria monocytogenes* with mammalian cells during entry and actin-based movement : bacterial factors , cellular ligands and signaling. *Embo Journal* 1998, 3797-3806.
11. Diagnostic Systems, B. D. Instructions for use - BD ™ PALCAM Listeria Agar. 2003, 1-4.

12. Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., Martin, P. Differentiation of the Major *Listeria monocytogenes* Serovars by Multiplex PCR. *Journal of Clinical Microbiology* 2004, 3819-3822.
13. Farber, J. M., Losos, J. Z. *Listeria monocytogenes* - a foodborne pathogen. *Canadian Medical Association Journal* 1988, 413-418.
14. Farber, J. M., Peterkin, P. I. *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews* 1991, 476-511.
15. Felício, M. T. S., Hogg, T., Gibbs, P., Teixeira, P., Wiedmann, M. Recurrent and Sporadic *Listeria monocytogenes* Contamination in Alheiras Represents Considerable Diversity, Including Virulence-Attenuated Isolates. *Applied and Environmental Microbiology* 2007, 3887-3895.
16. Ferreira, V.: Persistent *Listeria monocytogenes* in fermented meat sausage production facilities in Portugal represent diverse geno and phenotypes. 2010.
17. Ferreira, V., Barbosa, J., Stasiewicz, M., Vongkamjan, K., Moreno Switt, A., Hogg, T., Gibbs, P., Teixeira, P., Wiedmann, M. Persistent Listeria monocytogenes in fermented meat sausage production facilities in Portugal represent diverse geno- and phenotypes. *Applied and Environmental Microbiology* 2011, 2701-2715.
18. Freitag, N. E., Port, G. C., Miner, M. D. *Listeria monocytogenes* — from saprophyte to intracellular pathogen. *Nature Reviews Microbiology* 2009.
19. Gaillard, J., Berche, P., Frehei, C. Entry of *L . monocytogenes* into Cells Is Mediated by Internalin , a Repeat Protein Reminiscent of Surface Antigens from G ram-Positive Cocci. *Cell* 1991, 1127-1141.
20. Gaillard, J., Berche, P., Mounier, J., Sansonetti, P., Richard, S. In Vitro Model of Penetration and Intracellular Growth of *Listeria monocytogenes* in the Human Enterocyte-Like Cell Line Caco-2. *Infection and Immunity* 1987, 2822-2829.
21. Graves, L. M., Helsel, L. O., Steigerwalt, A. G., Morey, R. E., Daneshvar, M. I., Roof, S. E., Orsi, R. H., Fortes, E. D., Milillo, S. R., den Bakker, H. C. *et al*. *Listeria marthii* sp. nov., isolated from the natural environment, Finger Lakes National Forest. *International Journal of Systematic and Evolutionary Microbiology* 2010, 1280-1288.
22. Gray, M. I., Killinger, A. H. *Listeria monocytogenes* and listeric infections. *Bacteriological reviews* 1966, 309-382.
23. Gray, M. J., Freitag, N. E., Boor, K. J. How the Bacterial Pathogen Listeria monocytogenes Mediates the Switch from Environmental Dr . Jekyll to Pathogenic Mr . Hyde. *Infection and Immunity* 2006, 2505-2512.

24. Gray, M. J., Zadoks, R. N., Fortes, E. D., Dogan, B., Cai, S., Chen, Y., Scott, V. N., Gombas, D. E., Boor, K. J., Wiedmann, M. Listeria monocytogenes isolates from foods and humans form distinct but overlapping populations. *Applied and Environmental Microbiology* 2004, 5833-5841.
25. Handa-Miya, S., Kimura, B., Takahashi, H., Sato, M., Ishikawa, T., Igarashi, K., Fujii, T. Nonsense-mutated inlA and prfA not widely distributed in *Listeria monocytogenes* isolates from ready-to-eat seafood products in Japan. *International Journal of Food Microbiology* 2007, 312-318.
26. Jacquet, C., Doumith, M., Gordon, J. I., Martin, P. M. V., Cossart, P., Lecuit, M. A Molecular Marker for Evaluating the Pathogenic Potential of Foodborne *Listeria monocytogenes*. *Journal of Investigative Dermatology* 2004, 2094-2100.
27. Jeffers, G. T., Bruce, J. L., Mcdonough, P. L., Scarlett, J., Boor, K. J., Wiedmann, M. Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. *Microbiology* 2001, 1095-1104.
28. Jonquieres, R., Bierne, H., Mengaud, J., Cossart, P. The inlA Gene of *Listeria monocytogenes* LO28 Harbors a Nonsense Mutation Resulting in Release of Internalin. *Infection and Immunity* 1998, 3420-3422.
29. Kathariou, S.: Foodborne Outbreaks of Listeriosis and Epidemic-Associated Lineages of *Listeria Monocytogenes*. In: *Microbial Food Safety in Animal Agriculture.* Blackwell Publishing; 2008: 243-256.
30. Kathariou, S. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection* 2002, 1811-1829.
31. Leclercq, A., Clermont, D., Bizet, C., Grimont, P. A. D., Le Flèche-Matéos, A., Roche, S. M., Buchrieser, C., Cadet-Daniel, V., Le Monnier, A., Lecuit, M. *et al*. *Listeria rocourtiae sp. nov*. *International Journal of Systematic and Evolutionary Microbiology* 2010, 2210-2214.
32. Lecuit, M., Nelson, D. M., Smith, S. D., Khun, H., Huerre, M., Vacher-Lavenu, M.-C., Gordon, J. I., Cossart, P. Targeting and crossing of the human maternofetal barrier by *Listeria monocytogenes*: Role of internalin interaction with trophoblast E-cadherin. *Proceedings of the National Academy of Sciences of the United States of America* 2004, 6152-6157.
33. Lecuit, M., Ohayon, H., Braun, L., Mengaud, J., Cossart, P. Internalin of *Listeria monocytogenes* with an intact leucine-rich repeat region is sufficient to promote internalization. *Infection and Immunity* 1997, 5309-5319.
34. Lecuit, M., Ohayon, H., Braun, L., Mengaud, J., Cossart, P., Ohayon, N. E., Braun, L., Lecuit, M. Internalin of *Listeria monocytogenes* with an Intact

Leucine-Rich Repeat Region Is Sufficient To Promote Internalization. *Infection and Immunity* 1997, 5309-5319.

35. Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., Babinet, C., Cossart, P. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science (New York, NY)* 2001, 1722-1725.
36. Liu, D. Identification , subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *Journal of Medical Microbiology* 2006, 645-659.
37. McLauchlin, J., Mitchell, R. T., Smerdon, W. J., Jewell, K. *Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. *International Journal of Food Microbiology* 2004, 15-33.
38. Microgen, B.: Aloa Chromagenic Agar. In., vol. 44; 2007: 15-16.
39. Nightingale, K. *Listeria monocytogenes*: kwonledge gained through DNA Sequence-Based Subtyping, Implications and Future Considerations. *Microbiological methods* 2010, 1-12.
40. Nightingale, K. K., Ivy, R. A., Ho, A. J., Fortes, E. D., Njaa, B. L., Peters, R. M., Wiedmann, M. inlA Premature Stop Codons Are Common among *Listeria monocytogenes* Isolates from Foods and Yield Virulence-Attenuated Strains That Confer Protection against Fully Virulent Strains. *Applied and Environmental Microbiology* 2008, 6570-6583.
41. Nightingale, K. K., Windham, K., Martin, K. E., Yeung, M., Wiedmann, M. Select *Listeria monocytogenes* subtypes commonly found in foods carry distinct nonsense mutations in *inlA*, leading to expression of truncated and secreted internalin A, and are associated with a reduced invasion phenotype for human intestinal epithelial cells. *Applied and Environmental Microbiology* 2005, 8764-8772.
42. Olier, M., Garmyn, D., Rousseaux, S., Lemaître, J.-p., Piveteau, P., Guzzo, J. Truncated Internalin A and Asymptomatic *Listeria monocytogenes* Carriage : In Vivo Investigation by Allelic Exchange Truncated Internalin A and Asymptomatic *Listeria monocytogenes* Carriage : In Vivo Investigation by Allelic Exchange. *Infetion and Immunity* 2005, 644-648.
43. Olier, M., Pierre, F., Lemaı, J. P., Divies, C. Assessment of the pathogenic potential of two *Listeria monocytogenes* human faecal carriage isolates. *Microbiology* 2002, 1855-1862.

44. Orndorff, P. E., Hamrick, T. S., Smoak, I. W., Havell, E. A. Host and bacterial factors in listeriosis pathogenesis. *Veterinary Microbiology* 2006, 1-15.
45. Orsi, R. H., den Bakker, H. C., Wiedmann, M., Bakker, H. C. D., Wiedmann, M. *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *International journal of medical microbiology* 2011, 79-96.
46. Orsi, R. H., Ripoll, D. R., Yeung, M., Nightingale, K. K., Wiedmann, M. Recombination and positive selection contribute to evolution of *Listeria monocytogenes* inlA. *Microbiology* 2007, 2666-2678.
47. Orsi, R. H., Sun, Q., Wiedmann, M. Genome-wide analyses reveal lineage specific contributions of positive selection and recombination to the evolution of *Listeria monocytogenes*. *BMC Evolutionary Biology* 2008, 233-233.
48. Piffaretti, J. C., Kressebuch, H., Aeschbacher, M., Bille, J., Bannerman, E., Musser, J. M., Selander, R. K., Rocourt, J. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. *Proceedings of National Academy of Sciences* 1989, 3818-3822.
49. Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Le Monnier, A., Brisse, S. A new perspective on *Listeria monocytogenes* evolution. *Plos Pathogens* 2008.
50. Ramalheira, R., Almeida, M., Azeredo, J., Branda, T. R. S., Silva, J., Teixeira, P. Survival of Clinical and Food Isolates of *Listeria monocytogenes* Through Simulated. *Foodborne Pathogens And Disease* 2010.
51. Rasmussen, O. F., Skouboe, P., Dons, L., Rossen, L., Olsen, J. E. *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. *Microbiology* 1995, 2053-2061.
52. Roberts, A., Nightingale, K., Jeffers, G., Fortes, E., Kongo, J. M., Wiedmann, M. Genetic and phenotypic characterization of *Listeria monocytogenes* lineage III. *Microbiology* 2006, 685-693.
53. Roberts, A., Nightingale, K., Jeffers, G., Fortes, E., Kongo, J. M., Wiedmann, M., Roberts, A. Genetic and phenotypic characterization of *Listeria monocytogenes* lineage III. *Microbiology (Reading, England)* 2006, 685-693.
54. Roberts, A. J., Williams, S. K., Wiedmann, M., Nightingale, K. K., Icrobiol, A. P. P. L. E. N. M. Some Listeria monocytogenes Outbreak Strains Demonstrate Significantly Reduced Invasion , *inlA* Transcript Levels , and Swarming Motility In Vitro □. *Applied and Environment Microbiology* 2009, 5647-5658.

55. Roucourt, J., Buchrieser, C.: The Genus *Listeria* and *Listeria monocytogenes* Phylogenetic position, Taxonomy and Identification - *Listeria*, Listeriosis and Food Safety, 3rd edn. Boca Raton: E.T.Ryers and E.H. Marth; 2007
56. Rousseaux, S., Olier, M., Lemaître, J. P., Piveteau, P., Guzzo, J. Use of PCR-Restriction Fragment Length Polymorphism of *inlA* for Rapid Screening of *Listeria monocytogenes* Strains Deficient in the Ability To Invade Caco-2 Cells. *Applied and Environmental Microbiology* 2004, 2180-2185.
57. Sauders, B. D., Durak, M. Z., Fortes, E., Windham, K., Schukken, Y., Lembo, A. J., Akey, B., Nightingale, K. K., Wiedmann, M. Molecular Characterization of *Listeria monocytogenes* from Natural and Urban Environments. *Journal of Food Protection* 2006, 93-105.
58. Schubert, W. D., Urbanke, C., Ziehm, T., Beier, V., Machner, M. P., Domann, E., Wehland, J., Chakraborty, T., Heinz, D. W. Structure of internalin, a major invasion protein of *Listeria monocytogenes*, in complex with its human receptor E-cadherin. *Cell* 2002, 825-836.
59. Schuchat, A., Swaminathan, B., Broome, C. V. Epidemiology of Human Listeriosis. *Clinical Microbiology Reviews* 1991, 169-183.
60. Sousa, S., Cabanes, D., El-Amraoui, A., Petit, C., Lecuit, M., Cossart, P. Unconventional myosin VIIa and vezatin, two proteins crucial for *Listeria* entry into epithelial cells. *Journal of Cell Science* 2004, 2121-2130.
61. Swaminathan, B., Gerner-Smidt, P. The epidemiology of human listeriosis. *Microbes and Infection* 2007, 1236-1243.
62. Troyanovsky, S. Cadherin dimers in cell–cell adhesion. *European Journal of Cell Biology* 2005, 225-233.
63. Van Stelten, a., Nightingale, K. K. Development and implementation of a multiplex single-nucleotide polymorphism genotyping assay for detection of virulence-attenuating mutations in the *Listeria monocytogenes* virulence-associated gene *inlA*. *Applied and Environmental Microbiology* 2008, 7365-7375.
64. Van Stelten, a., Simpson, J. M., Ward, T. J., Nightingale, K. K. Revelation by single-nucleotide polymorphism genotyping that mutations leading to a premature stop codon in inlA are common among *Listeria monocytogenes* isolates from ready-to-eat foods but not human listeriosis cases. *Applied and Environmental Microbiology* 2010, 2783-2790.

65. Vázquez-Boland, J. A., Domínguez-Bernal, G., González-Zorn, B., Kreft, J., Goebel, W. Pathogenicity islands and virulence evolution in *Listeria*. *Microbes and Infection* 2001, 571-584.
66. Ward, T. J., Ducey, T. F., Usgaard, T., Dunn, K. A., Bielawski, J. P. Multilocus Genotyping Assays for Single Nucleotide Polymorphism-Based Subtyping of *Listeria monocytogenes* Isolates. *Applied and Environmental Microbiology* 2008, 7629-7642.
67. Ward, T. J., Evans, P., Wiedmann, M., Usgaard, T., Roof, S. E., Stroika, S. G., Hise, K. Molecular and Phenotypic Characterization of *Listeria monocytogenes* from U.S. Department of Agriculture Food Safety and Inspection Service Surveillance of Ready-to-Eat Foods and Processing Facilities. *Journal of Food Protection* 2010, 861-869.
68. White, S. L.: Virulence Attenuation and Relative Fitness of *Listeria monocytogenes* from the Processing Plant Environment. North Carolina State University; 2010.
69. Wiedmann, M. Molecular Subtyping Methods for *Listeria monocytogenes*. *World Health* 2002, 524-532.
70. Yu, W. L., Dan, H., Lin, M. InlA and InlC2 of *Listeria monocytogenes* serotype 4b are two internalin proteins eliciting humoral immune responses common to listerial infection of various host species. *Current Microbiology* 2008, 505-509.